Inhibitory effect of liposome-entrapped lemongrass oil on the growth of *Listeria monocytogenes* in cheese

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ABSTRACT

Listeria monocytogenes infection in dairy products is of mounting public concern. To inhibit bacterial growth, we engineered stimuli-responsive liposomes containing lemongrass oil for this study. The controlled release of liposome-entrapped lemongrass oil is triggered by listerolysin O, secreted by *L. monocytogenes*. We investigated the antibiotic activities of lemongrass oil liposomes against *L. monocytogenes* in cheese. We also assessed their possible effects on the quality of the cheese. Liposomes containing lemongrass oil (5.0 mg/mL) presented the optimal polydispersity index (0.246), zeta-potential (−58.9 mV) and entrapment efficiency (25.7%). The liposomes displayed satisfactory antibiotic activity against *L. monocytogenes* in cheese over the storage period at 4°C. We observed no effects on the physical and sensory properties of the cheese after the liposome treatment.

Key words: listerolysin O, lemongrass oil liposome, *Listeria monocytogenes*, cheese

INTRODUCTION

Dairy products such as cheese play an important nutritional role in human health, because they contain a range of nutrients, including proteins, vitamins, and minerals (Songisepp et al., 2012). However, many dairy products, especially cheeses, are made from raw milk, increasing the risk of contamination by foodborne pathogens (Jamali et al., 2015). *Listeria monocytogenes*, one of the main pathogenic bacteria found in cheese, is remarkable because it is associated with high mortality rates (Lee et al., 2014). Furthermore, *L. monocytogenes* has the ability to adapt to various environments and extreme conditions, including refrigeration temperatures, water activity (<0.94), and low pH (<4.4) (Rückerl et al., 2014). The incidence of *L. monocytogenes* contamination in cheese is 7.4% on average, but ranges from 1%

to 22% (Guenther and Loessner, 2011). For this reason, *L. monocytogenes* is an important challenge for cheese production.

A number of artificial additives have been applied during cheese production to minimize the risk of *L. monocytogenes* contamination, but concerns have been raised about their potential toxicity and cancer-causing effects (Tepe et al., 2005). In that context, it is desirable to develop and use natural additives with a good safety profile and excellent antibiotic activity. Essential oils obtained from aromatic plants are of great interest because of their outstanding antibacterial and antioxidative properties (Ahmad and Viljoen, 2015).

Lemongrass (*Cymbopogon citratus*) has seen widespread use in folk medicine as a treatment for many disorders and infectious diseases (Balakrishnan et al., 2014). Lemongrass oil (LO) has been reported to possess excellent antibiotic activity against a considerable number of microorganisms, including bacteria, yeast, and fungi (Azarakhsh et al., 2014). However, high volatility, photosensitivity and low solubility have limited the use of essential oils in food preservation (Kim et al., 2014). Incorporating essential oils into liposome systems could reduce these effects and prolong valid antibacterial time.

Liposomes are made up of one or more concentric phospholipid bilayers and an internal aqueous phase (Sebaaly et al., 2015). Because of their nontoxic, biocompatible, and nonimmunogenic properties, liposomes are widely used to encapsulate volatile and lipophilic ingredients such as vitamin E and essential oils (Tonizazzo et al., 2014). Recently, a new generation of stimuli-responsive liposomes have been developed. They are focused on the intelligent release of encapsulated bioactive molecules, triggered by various stimulating agents (Kenneth et al., 2007). One of these stimulating agents is pore-forming toxin (Mueller et al., 2009; Lin et al., 2015).

Listerolysin O is a cholesterol-dependent pore-forming toxin secreted by many gram-positive bacteria, but *L. monocytogenes* is the only foodborne pathogen that secretes listerolysin O inside cells (Hamon et al., 2006). This toxin can form large pores on the phospholipidic

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bilayers of liposomes and trigger the release of antibiotic substances encapsulated in the liposomes, inactivating *L. monocytogenes* (Schuerch et al., 2005).

This controlled-release mechanism, triggered by pore-forming toxins, carries far-reaching implications for future food preservation, because food decay and potential foodborne diseases are caused mainly by pathogens. The purpose of this study was to investigate the antibiotic performance of liposome-entrapped LO against *L. monocytogenes* in cheese and the potential effects of LO on the physical and sensory attributes of the cheese itself.

MATERIALS AND METHODS

Materials and Culture

Lemongrass oil was purchased from J.E. International (Caussols, France). Fresh hard cheese (Kerrygold Cheddar) was bought from a local supermarket. Soy lecithin, cholesterol, chloroform, and polyvinylpyrrolidone were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, P. R. China).

Listeria monocytogenes ATCC 19115 (China General Microbiological Culture Collection Center, Beijing, P. R. China) was stored in liquid paraffin wax at 4°C and cultured with shaking at 37°C for 48 h.

LO Encapsulation

Lemongrass oil was entrapped in a liposome system using the method described by Cui et al. (2016) with some modifications. Initially, LO (0, 3, 4, 5, and 6 mg/mL), soy lecithin (20 mg/mL), and cholesterol (4 mg/mL) were mixed in chloroform, and the solvent was removed by rotary evaporation at 35–40°C. The obtained thin film was placed in a vacuum oven at 60°C overnight. Then, polyvinylpyrrolidone (1 mg/mL) was mixed and hydrated in PBS (0.03 M, pH 7.2). The hydration solution was homogenized by an ultrasonicator (Ymnl-1000Y, Nanjing Immanuel Instrument Equipment Co. Ltd., Nanjing, P. R. China) for 35 min. After that, the LO liposome system was centrifuged for 10 min at $2,624 \times g$. Finally, the LO liposomes were filtered using a 0.22- μ m filter membrane.

Characterization of LO Liposomes

We characterized LO liposomes in terms of particle size, polydispersity index (**PDI**), surface zeta-potential, and entrapment efficiency (**EE**). We used a dynamic light scattering zetasizer (Nano ZS90, Malvern Instruments, Worcester, UK) to determine particle size, PDI, and surface zeta-potential. We measured EE as

described in a previous study (Sebaaly et al., 2015). We analyzed different concentrations of LO (0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL, 1.6 mg/mL, and 3.2 mg/mL) using GC-MS (Agilent 6890N/5973N, Agilent Technologies, Santa Clara, CA). We obtained the peak areas of geranial and neral (the main chemical components of LO) and constructed a standard curve for geranial (peak area versus concentration). The LO liposomes were dissolved in ethanol and the compounds were detected by GC-MS. All experiments were carried out 3 times.

We calculated EE using the following formula:

$$EE (\%) = \frac{A}{B} \times 100,$$

where A = concentration of encapsulated LO (mg/mL); B = concentration of free LO added before encapsulation (mg/mL).

Antibiotic Activity of LO Liposomes Against *L. monocytogenes* In Vitro

We carried out a kill-time analysis to estimate the antibiotic activity of LO liposomes. We dissolved LO liposomes in PBS to concentrations of 2.5% (vol/vol), 5.0%, and 10%. The PBS samples containing empty liposomes (5%) and free LO (0.01%) were used as controls. Next, we added cell suspensions of *L. monocytogenes* to the LO liposome/PBS solutions and controls. The samples had an initial bacterial concentration of 10^5 cfu/mL and were confluent and cultured at 37°C. The number of residual bacteria were counted at 0, 24, 48, and 72 h. All experiments were carried out 3 times via the method described by Cui et al. (2015).

Four kinds of samples were prepared, including LO (0.01%), LO liposomes (2.5%), PBS and LO liposomes (2.5%) incubated with *L. monocytogenes* for 48 h. Then, they were analyzed by an Agilent 7890A gas chromatograph (Agilent Technologies) with an HP-5 capillary column attached. The amount of the injection was 1 μ L. The injector and detector temperatures were 250 and 230°C, respectively (Ahmad and Viljoen, 2015).

Antibiotic Activity of LO Liposomes Against *L. monocytogenes* in the Model Cheese Suspension

Cheese (10 g) and water (90 g) were blended into a slurry and sterilized. The LO liposomes were added to the cheese suspension containing *L. monocytogenes* (approximately 10^3 cfu/mL) in different concentrations (10, 20, 30, and 40%). The samples containing empty liposomes (40%) and free LO (0.05%) were used as

Table 1. Characteristics (means $\pm$ SD) of liposomes with different lemongrass oil (LO) encapsulations

LO (mg/mL)	Particle size (nm)	Polydispersity index	Zeta-potential (mV)	Entrapment efficiency (%)
0	63.6 $\pm$ 1.76	0.235 $\pm$ 0.023	-24.8 $\pm$ 2.38	0
3	145.7 $\pm$ 2.97	0.304 $\pm$ 0.031	-54.3 $\pm$ 1.55	18.1 $\pm$ 1.66
4	163.5 $\pm$ 2.45	0.281 $\pm$ 0.043	-55.6 $\pm$ 0.96	21.5 $\pm$ 1.09
5	188.1 $\pm$ 1.82	0.246 $\pm$ 0.016	-58.9 $\pm$ 3.40	25.7 $\pm$ 0.97
6	196.8 $\pm$ 3.54	0.269 $\pm$ 0.028	-53.4 $\pm$ 1.32	22.4 $\pm$ 1.43

controls. Samples were cultured at 4 and 25°C, and we monitored the amount of change in *L. monocytogenes* for 7 and 14 d. All experiments were carried out 3 times (Cui et al., 2015).

Antibiotic Activity of LO Liposomes in Cheese

Cheese samples (3.5 $\times$ 3 $\times$ 1 cm) were inoculated with *L. monocytogenes* suspension to obtain an initial bacterial concentration of 10³ cfu/g, and then samples were maintained for 10 min at room temperature under sterile conditions. The inoculated samples were sprayed with LO liposomes (40%) to a dose of 1 mL/100 g, or with empty liposomes or free LO at equivalent doses. All samples were packaged in sterile bags and stored at 4°C for 15 d. Inoculated cheese samples with no treatments were tested as controls. We performed microbial analysis using *Listeria* Chromogenic Medium (Huankai Microbial Technology Co. Ltd., Guangzhou, P. R. China) and plate colony counting. All experiments were carried out 3 times.

Food Physical Quality Analysis

To assess the possible effects of LO liposomes on cheese, we measured the physical qualities (surface color and texture) of the cheese samples. Fresh cheese samples (3.5 $\times$ 3 $\times$ 1 cm) without *L. monocytogenes* treatment were sprayed with LO liposomes to a dose of 1 mL/100 g. Samples without LO liposomes were used as controls. All samples were packaged in sterile bags and stored at 4°C for 15 d. Surface-color analysis was carried out using a chromatic meter (Color Quest XE, HunterLab, Reston, VA) in the dark, including L* (lightness), a* (redness), and b* (yellowness) values. The measured aperture size of the chromatic meter was 19.0 mm. We used a xenon lamp as an illuminant. The standard observer angle was 8°. The instrument was calibrated using a standard white plate before measurement.

We analyzed the texture properties (hardness, springiness, cohesiveness, gumminess, and chewiness) of the cheese samples (diameter: 3 cm; thickness: 2 cm) using a texture analyzer (TA.XT. Plus, Stable Micro Systems

Ltd., Godalming, UK). Samples were compressed twice at a speed of 2.00 mm/s and a trigger force of 0.005 kg. All experiments were carried out 3 times.

Sensory Evaluation

Twenty untrained panelists (students and staff at Jiangsu University) evaluated the sensory attributes of the cheese samples. During the sensory evaluation, untreated and LO liposome-treated samples were placed randomly on uniform white coded plates. Participants scored color, taste, texture, off-flavor, and overall acceptability using a 5-point hedonic scale (1, dislike very

Figure 1. Size distribution (a) and surface zeta-potentials (b) of liposomes containing lemongrass oil (5 mg/mL). All experiments in this study were carried out 3 times; error bars indicate the potential error of the data. Color version available online.

much, to 5, like very much; Ulpathakumbura et al., 2016).

Statistical Analysis

We used SPSS software (version 22.0; IBM Corp., Armonk, NY) to analyze the results. Data were reported as mean $\pm$ SD.

RESULTS AND DISCUSSION

Characterization of LO Liposomes

The characteristics of the LO liposomes are displayed in Table 1. Initially, the mean particle size ranged from 145.7 ± 2.97 nm to 196.8 ± 3.54 nm. Compared with empty liposomes (63.6 ± 1.76 nm), the diameter of liposomes was higher after encapsulating LO. Size is a critical indicator for liposomes. Small particles (diameter <200 nm) have the desired physicochemical properties and bioavailability for stable performance that leads to the sustained release of encapsulated active substances (Wang et al., 2010). Our results revealed

that LO liposomes had a suitable size range and could maintain stability.

The PDI values for empty liposomes ranged from 0.235 ± 0.023 to 0.304 ± 0.031 , indicating a relatively narrow distribution of particle size.

Surface zeta-potential values for LO liposomes in re-distilled water ranged from -53.4 ± 1.32 mV to -58.9 ± 3.40 mV, and for empty liposomes was -24.8 ± 2.38 mV. Conventionally, zeta-potential values of less than -30 mV and more than $+30$ mV are considered to indicate good stability, avoiding aggregation or precipitation (Lu et al., 2014; Natrajan et al., 2015). Therefore, the LO liposome suspension could be a stable system compared with empty liposomes.

The EE of LO liposomes was analyzed using GC-MS. The linear equation of LO standard curve was as follows: $y = 33,730,030.3427x + 801,175.3750$ ($R^2 = 0.9995$). According to the linear equation, the EE of LO in the liposome system ranged from $18.1 \pm 1.66\%$ to $25.7 \pm 0.97\%$ (Table 1). The liposome EE peaked at a 5 mg/mL of LO, declining slightly at a concentration of 6 mg/mL. However, EE values for LO liposomes were low. During measurement of EE, a small proportion of

Figure 2. (a) Kill-time analysis of lemongrass oil (LO) liposomes for *Listeria monocytogenes*; (b) gas chromatogram of LO (0.01%), LO liposomes (2.5%), PBS and LO liposomes (2.5%) incubated with *L. monocytogenes*. All experiments in this study were carried out 3 times; error bars indicate the potential error of the data.

the LO liposomes could have remained in the supernatant after centrifugation, resulting in loss of LO.

Based on the above analysis, we chose LO at a concentration of 5 mg/mL to engineer liposomes for further study because this concentration had the desired PDI, zeta-potential, and EE. The particle size distribution and zeta-potential of the LO liposomes (5 mg/mL) are displayed in Figure 1.

Antibiotic Activity of LO Liposomes Against *L. monocytogenes* In Vitro

Figure 2a displays the results of the kill-time analysis of LO liposomes on *L. monocytogenes* in PBS. The num-

ber of *L. monocytogenes* in empty liposomes samples increased slightly during incubation compared with controls. We found that free LO (0.01%) inhibited the growth of *L. monocytogenes* after 24 h of incubation, but that the number of *L. monocytogenes* increased after 48 h because free LO was highly volatile and was lost over the incubation period. We also observed that LO liposomes at concentrations of 10% significantly inhibited the growth of *L. monocytogenes* after 24 h of incubation. Finally, we found 99.4% and 99.999% reductions in population at concentrations of 2.5 and 5.0% after 48 h of incubation, respectively. The results indicated that LO liposomes could achieve the desired inhibitory effect against *L. monocytogenes* in vitro.

Figure 3. Antibacterial activity of lemongrass oil (LO) liposomes [PBS control, empty liposomes (40%), LO (0.05%), LO liposomes (10, 20, 30, 40%)] against *Listeria monocytogenes* in the model cheese suspension at (a) 25°C, and (b) 4°C. All experiments in this study were carried out 3 times; error bars indicate the potential error of the data.

Table 2. Antibiotic activity (log cfu/g; means $\pm$ SD) of lemongrass oil (LO) liposomes against *Listeria monocytogenes* in cheese during storage at 4°C

Treatment	Storage period (d)					
	0	3	6	9	12	15
Control	3.02 $\pm$ 0.57	3.56 $\pm$ 0.43	4.39 $\pm$ 0.76	5.45 $\pm$ 0.55	6.12 $\pm$ 0.29	6.58 $\pm$ 0.41
Empty liposomes	3.02 $\pm$ 0.57	3.67 $\pm$ 0.26	4.42 $\pm$ 0.48	5.53 $\pm$ 0.34	6.23 $\pm$ 0.52	6.63 $\pm$ 0.29
LO	3.02 $\pm$ 0.57	2.99 $\pm$ 0.36	3.96 $\pm$ 0.43	5.17 $\pm$ 0.21	6.16 $\pm$ 0.37	6.64 $\pm$ 0.45
LO liposomes	3.02 $\pm$ 0.31	2.89 $\pm$ 0.12	3.01 $\pm$ 0.41	2.96 $\pm$ 0.17	2.86 $\pm$ 0.32	2.78 $\pm$ 0.39

Results from the partial gas chromatography are shown in Figure 2b. We found no characteristic peaks of geranial and neral in the samples of PBS and LO liposomes (2.5%) solution without *L. monocytogenes*, showing that LO had practically no leakage from the liposome system. However, we did observe the 2 characteristic peaks in the liposomes incubated with *L. monocytogenes*, demonstrating that the prepared liposomes could intelligently release the antibacterial ingredient and effectively inhibit the growth of the bacteria. The results also indicated that the release of LO liposomes could be triggered by listerolysin O and lead to the leakage of LO, consistent with our hypothesis.

Antibiotic Activity of LO Liposomes Against *L. monocytogenes* in the Model Cheese Suspension

Results for the suppressive effects of LO liposomes on *L. monocytogenes* in the model cheese suspension at 25°C and 4°C are displayed in Figure 3. The number of *L. monocytogenes* generally increased with incubation time. The number of *L. monocytogenes* in the empty liposomes samples were slightly higher than in controls during the incubation period. The number of *L. monocytogenes* in the LO (0.05%) samples increased slowly on the first and second days, and then rapidly after 48 h because of the high volatility of LO. We also observed a positive correlation between the the concentration of LO liposomes and the inhibitory effect on *L. monocytogenes* during the incubation period. After 1d of incubation at 25°C, the numbers of bacteria treated by the liposomes (10, 20, and 30%) were increased by 3.78,

3.56, and 3.00 log₁₀ steps respectively, but those treated by the liposomes (40%) were increased by only 1.65 log₁₀ steps. Furthermore, the liposomes (40%) showed distinctly antibacterial activity against *L. monocytogenes* throughout the incubation period compared with other concentrations. Similar results were also demonstrated in the cheese suspension incubated at 4°C. The LO liposomes at high concentration demonstrated antilisterial activity as a result of the proteins and fat in the cheese suspension; those components could affect the antibiotic activities of the liposomes (Liu and Yang, 2012). Therefore, we chose LO liposomes (40%) for further antimicrobial and quality analyses.

Antibiotic Activity of LO Liposomes in Cheese

Table 2 displays the antibiotic activity of LO liposomes against *L. monocytogenes* in cheese during storage at 4°C. Generally, *L. monocytogenes* growth in the cheese blocks was slower than in the cheese suspension at the same temperature, possibly because of lower moisture in the cheese blocks. The numbers of *L. monocytogenes* in the control samples increased steadily over the storage period and reached 6.58 log cfu/g at 15 d. The number of *L. monocytogenes* in empty liposome samples was slightly higher than control samples during the incubation period. The number of *L. monocytogenes* in LO (5%) samples increased slowly on the third day, but rapidly after 3 d because of the high volatility of LO. However, bacteria counts were markedly inhibited by LO liposomes (40%). The results indicated that LO liposomes showed positive inhibitory activity against *L.*

Table 3. Changes in surface-color values (means $\pm$ SD) of cheese samples with or without lemongrass oil liposome treatment during storage at 4°C

Color properties	Fresh		15 d	
	Control	Liposomes	Control	Liposomes
L*	72.457 $\pm$ 0.012	71.523 $\pm$ 0.033	70.233 $\pm$ 0.043	70.102 $\pm$ 0.077
a*	0.847 $\pm$ 0.017	0.896 $\pm$ 0.025	0.824 $\pm$ 0.029	0.811 $\pm$ 0.054
b*	24.573 $\pm$ 0.069	25.147 $\pm$ 0.019	25.846 $\pm$ 0.031	25.598 $\pm$ 0.067

Table 4. Texture analysis (means $\pm$ SD) of cheese samples with or without lemongrass oil liposome treatment during storage at 4°C

Texture parameters	Fresh		15 d	
	Control	Liposomes	Control	Liposomes
Hardness (kg)	22.61 $\pm$ 1.06	21.08 $\pm$ 1.32	24.13 $\pm$ 0.93	23.84 $\pm$ 1.15
Cohesion (%)	0.567 $\pm$ 0.001	0.559 $\pm$ 0.001	0.586 $\pm$ 0.002	0.599 $\pm$ 0.001
Springiness (mm)	0.83 $\pm$ 0.39	0.86 $\pm$ 0.24	0.90 $\pm$ 0.27	0.92 $\pm$ 0.33
Gumminess (kg)	23.29 $\pm$ 0.14	22.97 $\pm$ 0.22	24.66 $\pm$ 0.16	24.41 $\pm$ 0.19
Chewiness (kg)	12.45 $\pm$ 0.56	12.33 $\pm$ 0.77	13.35 $\pm$ 0.45	13.29 $\pm$ 0.61

monocytogenes in cheese blocks, and that LO liposomes could be a promising antiseptic for cheese preservation.

Food Physical Quality Analysis

The surface-color values of the cheese samples with or without LO liposomes during the storage period are displayed in Table 3. Generally, L* and a* values decreased and the b* values increased. The L*, a*, and b* values of cheese samples treated with the liposomes fluctuated slightly compared with controls, but we observed no significant differences between them over the same storage time. The results demonstrated that the surface color of the samples was not affected by the liposome treatment.

The results of the texture parameter analysis are presented in Table 4. During the storage period, the texture property values increased slightly for all samples; this may have been caused by lower moisture content during storage time (Youssef et al., 2015). However, we observed no significant differences in textural parameters between the control and liposome-treated samples over same storage time. The results showed that the cheese texture was barely altered by LO liposome treatment.

Sensory Evaluation

Sensory properties such as aroma and taste are crucial features of cheese, affecting consumers' purchas-

ing decisions. The results of the sensory evaluation are shown in Table 5. The sensory score of the cheese samples declined slightly over the storage period, but we observed no significant differences between the control and LO liposome-treated samples. Generally, the sensory quality of the cheese samples was maintained with LO liposome treatment.

CONCLUSIONS

This work investigated the antilisterial activity of liposomes containing LO on cheese. Desired antibiotic activity against *L. monocytogenes* was achieved after treatment with LO liposomes without affecting the quality of the cheese samples. Correspondingly, the shelf life of the cheese samples was extended markedly. Liposomal encapsulation technology enriches the possible application of essential oils in the dairy industry. Liposome-entrapped essential oils may have a place in the fight against foodborne pathogens.

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Table 5. Sensory evaluation of cheese samples with or without lemongrass oil liposome treatment during storage at 4°C

Sensory parameter ¹	Fresh		15 d	
	Control	Liposomes	Control	Liposomes
Color	2.87 $\pm$ 0.43	2.84 $\pm$ 0.22	2.67 $\pm$ 0.61	2.63 $\pm$ 0.37
Taste	2.56 $\pm$ 0.38	2.53 $\pm$ 0.16	2.47 $\pm$ 0.54	2.42 $\pm$ 0.49
Texture	2.66 $\pm$ 0.51	2.61 $\pm$ 0.64	2.49 $\pm$ 0.28	2.45 $\pm$ 0.43
Off-flavor	2.81 $\pm$ 0.19	2.78 $\pm$ 0.23	2.72 $\pm$ 0.43	2.67 $\pm$ 0.51
Overall acceptability	2.72 $\pm$ 0.35	2.77 $\pm$ 0.27	2.63 $\pm$ 0.22	2.59 $\pm$ 0.46

¹Data are the means ($\pm$ SD) of a 5-point hedonic scale: 1 = dislike very much; 2 = dislike somewhat; 3 = neither like nor dislike; 4 = like somewhat; 5 = like very much.

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